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O'Hare, E., Jeggo, R., Kim, E-M., Barbour, B., Walczak, J-S., Palmer, P., Lyons, T., Page, D., Hanna, D., Meara, J. R., Spanswick, D., Guo, J-P., McGeer, E. G., McGeer, P. L., & Hobson, P. (2016). Lack of support for bexarotene as a treatment for Alzheimer's disease. *Neuropharmacology*, *100*, 124-130.

Published in:
Neuropharmacology

Document Version:
Peer reviewed version

Neuropharmacology

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Abstract

Bexarotene has been reported to reduce brain amyloid-β (Aβ) levels and to improve cognitive function in transgenic mouse models of Alzheimer's disease (AD). Four groups failed to fully replicate the primary results but the original authors claimed overall support for the general conclusions. Because of its potential clinical importance, the current work studied the effects of bexarotene using two animal species and highly relevant paradigms. Rats were tested for the ability of bexarotene to prevent changes induced by an Aβ challenge in the form intracerebroventricular (i.c.v) administration of 7PA2 conditioned medium (7PA2 CM) which contains high levels of Aβ species. Bexarotene had no effect on the long-term potentiation of evoked extracellular field excitatory postsynaptic potentials induced by i.c.v. 7PA2 CM. It also had no effect following subcutaneous administration of 2, 5, 10 and 15 mg/kg on behavioral/cognitive impairment using an alternating-lever cyclic-ratio schedule of operant responding in the rat. The effects of bexarotene were further tested using the APPSwFILon,PSEN1*M146L*L286V transgenic mouse model of AD, starting at the time Aβ deposits first begin to develop. Mice were sacrificed after 48 days of exposure to 100 mg bexarotene per day. No significant difference between test and control mice was found using a water-maze test, and no significant difference in the number of Aβ deposits in cerebral cortex, using two different antibodies, was apparent. These results question the potential efficacy of bexarotene for AD treatment, even if instigated in the preclinical period prior to the onset of cognitive deficits reported for human AD.

KEY WORDS: Bexarotene; Alzheimer's disease; Beta-amyloid; Oligomers; Synaptic transmission; Behavior.

1. Introduction

Cramer et al. (2013) reported that bexarotene (Bex) administration rapidly decreased $A\beta_{1-40}$ and $A\beta_{1-42}$ interstitial fluid levels in the brains of two month old PPswe/PS1Δe9 (APP/PS1) transgenic (Tg) mice - a well-studied Tg mouse model of Alzheimer's disease (AD). The effect was seen within 6 hours of administration, and there was a 25% reduction of Aβ by 24 hours. A single dose of 100 mg/kg orally was reported to maintain the effect for 70 hours, and a similar outcome was found in normal (non-Tg) C57B1/6 mice. It was further reported that acute administration of Bex resulted in the rapid elimination of both diffuse and compact Aβ plaques in the cortex and hippocampus of the APP/PS1 mice. In 11 month old APP/PS1 mice, Bex administration for 7 days produced significantly reduced levels of soluble and insoluble $A\beta_{1-40}$ and $A\beta_{1-42}$, resulting in a 50% reduction in Aβ plaque numbers. Behaviourally, Bex was reported to restore cognition and memory in APP/PS1, APPPS1-21 and Tg2576 Tg mice. In addition, 90 days of Bex administration in APP/PS1 mice, and 20 days of administration in APPPS1-21 mice induced improved hippocampal function, as measured using a contextual fear conditioning task and a water maze task. Also, nest construction in the Tg2576 Tg mouse was restored after 72 hours of Bex administration, and odor habituation behavior was improved after 9 days of Bex administration when given to 12 and 14 month old mice. These findings implied that even acute administration of Bex to AD patients might be effective in treating both the early and late stages of AD.

Four independent research groups have already questioned these findings. Fitz et al., (2013), while confirming the observation of reversal of memory deficits and the decrease in interstitial fluid Aβ levels following Bex administration, found no effect on brain Aβ deposition. Veeraraghavalu et al. (2013) also found no effect of Bex in reducing the Aβ plaque burden in APP/PS1, 5XFAD, and APPPS1-21 mice. Price et al. (2013), using an almost identical treatment regimen to Cramer et al. (2013) were unable to find any evidence

for positive effects of Bex. Tesseur et al. (2013) using mice and dogs, also could not fully replicate the reported effects of Bex administration. All of these studies involved administering single doses or very short term daily doses of Bex by oral gavage. Shortly after, a further group using an almost identical treatment regimen was unable to find any evidence for positive effects of Bex (LaClair et al., 2013). In response to these reports, the original authors stated their data "replicate and validate our central conclusion that bexarotene stimulates the clearance of soluble β-amyloid peptides and results in the reversal of behavioral deficits in mouse models of Alzheimer's disease" (Landreth et al., 2013).

Because of the obvious lack of unambiguous confirmation of the effects of Bex relative to the treatment of AD, and the potential that the original report might have for clinical applications, we have further investigated the effects of Bex using more thorough paradigms. Two animal species and different but highly relevant models of AD were employed. One of the species utilized was normal male Sprague-Dawley rats. We administered into freely moving normal rats conditioned medium (CM) from 7PA2 cells by intracerebroventricular (i.c.v.) injection. 7PA2 CM expresses human amyloid precursor protein and secretes Aβ oligomers, dimers, trimers and tetramers (Podlisny et al., 1995; Podlisny et al., 1998; Walsh et al., 2002; Walsh et al., 2005). The CM contains a total human Aβ concentration in the $pg.ml^{-1}$ range, which is similar to that found in human CSF (Podlisny et al., 1998). The i.c.v. 7PA2 CM injection model has consistently been shown to impair LTP in experimental animals (e.g., Walsh et al., 2002; Townsend et al., 2006; O'Hare et al., 2013), and is therefore highly suitable to test any ameliorative effects of Bex on synaptic transmission. Effects were measured by recording long-term potentiation (LTP) of evoked extracellular field excitatory postsynaptic potentials (fEPSPs) in the CA1 region of hippocampal slices. The i.c.v. 7PA2 CM injection model has also consistently been shown to detrimentally affect memory-related behavior in the rat under an alternating-lever cyclic-ratio (ALCR) schedule of food

reinforcement (e.g., Cleary et al., 2005; Poling et al., 2008; Reed et al., 2011). Consequently, this method was used to assess the effect of Bex on behavior/cognition following subcutaneous (s.c.) administration of 2, 5, 10 and 15 mg/kg Bex in the rat.

The second species used was Tg mice. Tg AD mouse models have become a standard for screening potential pharmacological agents to treat AD. They have in common the introduction of one or more transgenes that enhance the production of human Aβ species. In view of the dramatic effects of Bex as reported by Cramer et al. (2013), it seemed appropriate to test the effects of Bex over time using an aggressive Tg mouse model of AD. The Jackson Laboratory Tg model of AD, APPSwFILon,PSEN1*M146L*L286V (Jackson Laboratory, Bar Harbor, ME, USA; B6SJSL) was employed as it is the most aggressive Tg mouse model of AD so far developed. Bex treatment was instigated at 7-8 weeks of age, when Aβ deposits first begin to develop in this model of AD. This would be analogous to treating AD cases in the preclinical phase of the disease, before cognitive deficits appear. Chronic administration of a very high dose of Bex (ca. 100 mg/kg/d) was used, as early treatment with such a high dose would be the best way of determining if Bex could have a preventative effect on Aβ accumulation and subsequent AD pathology. No ameliorative effects of Bex was found in any of the rat models (electrophysiological and behavioral), or mouse models (behavioral and histopathological) employed in the studies conducted.

2. Methods

2.1 *7PA2 cells*

7PA2 cells are stably transfected Chinese hamster ovary (CHO) cells which incorporate the cDNA for APP (APP751). This is specific for the familial AD mutation Val1717Phe (Podlisny et al., 1998; Shankar et al., 2011). The cells secrete $A\beta_{1-40}$ and $A\beta_{1-42}$ (Shankar et al., 2011). These cells were grown to just below confluence in DMEM containing 10% FBS

and 200 µg/ml G418. They were briefly washed in DPS and incubated at 37ºC with 5% CO_2 for 18 h with a sufficient volume of DMEM to cover the cells. After incubation, the medium was centrifuged at 3000 g for 15 min and either used directly or snap frozen and stored at -20ºC for later use. Using ELISA methodology, the concentration of total Aβ in the 7PA2 CM was in the range of 2-5 nM.

2.2 *Animals*

All animals were held in *vivaria* with automatically controlled temperature maintained at 23 ºC under a 12 h light/dark schedule (lights on at 0800 h). Specific details are given below for each manipulation. The animal experiments were conducted with the approval of the appropriate institutional ethics committees, and approved by the Animal Use Protocol 09-AOU-E-033 Canada, and license from the United Kingdom Home Office.

2.3 *LTP*

Evoked extracellular field excitatory postsynaptic potentials (fEPSPs) were recorded from 400 µm para-sagittal hippocampal slices prepared from male Sprague-Dawley rats (Charles River, Canada). After a 1 h recovery period at room temperature in artificial cerebrospinal fluid (aCSF; composition in mM: NaCl 127, KCl 1.6, KH_2PO_4 1.24, $MgSO_4$ 1.3, $CaCl_2$ 2.4, $NaHCO_3$ and D-glucose 10), the hippocampal slices were transferred to an interface chamber warmed to 30 ± 1ºC and perfused with aCSF. Schaffer collateral fibres were stimulated every 30 sec using a concentric bipolar electrode (FHC, USA) and evoked fEPSPs were recorded from the stratum radiatum of the CA1 region of the hippocampus. Stimulation intensity was set to evoke fEPSPs of 30-40% of the maximum amplitude. A 10 min stable baseline was recorded and thereafter the test substances were administered in aCSF for a 30 min period, prior to high frequency stimulation (HFS; 100 Hz for 1 sec) of the Schaffer collateral

pathway. The test substances used were 7PA2 CM (challenge) and CHO CM (wild-type control). Bex (Sigma, UK) was the test substance and DMSO the control vehicle. Bex was diluted with aCSF immediately prior to application to the hippocampal slices, giving final concentrations of 20 μM Bex and 0.1% DMSO. fEPSPs were recorded for 60 min after HFS stimulation, and the final 10 min of recording (20 sweeps) was used for group comparisons of LTP magnitude using a one-way ANOVA and Dunnett's post hoc test.

2.4 *Operant behavior test*

Behavioral data were collected using the ALCR schedule. Male Sprague-Dawley rats, weighing 220-250 g at the beginning of the experiment were maintained at 90% of their free-feeding body weights and housed individually with water available ad libitum. The rats were trained and tested in two-lever rat test chambers (Med Associates Inc., USA) enclosed in sound attenuating compartments. Food reinforcers were 45 mg sucrose pellets (BioServ, USA), that were delivered into a tray situated midway between the two operant response levers. The training regimen has been previously reported (Cleary et al., 2005; Reed et al., 2011). Briefly, operant behavioral sessions were conducted 7 d/wk and the rats were trained to press both levers for food reinforcement. Over approximately 20-30 sessions, the ALCR schedule of food reinforcement was introduced. Under this behavioral assay, rats must alternate to the other lever after pressing the currently correct lever a sufficient number of times to obtain a reinforcer. The number of lever presses required for each reinforcer changes, increasing sequentially from 2 responses per food pellet to 56 responses per food pellet, then decreasing sequentially from 56 responses per food pellet back to 2, and this sequence is repeated over 6 cycles. One complete cycle (of the 6) requires alternating lever responses of 2, 6, 12, 20, 30, 42, 56, 56, 42, 30, 20, 12, 6, and 2. This provides a measure of short-term memory, and generates data on two types of errors. These are lever-switching

errors, which occur when a subject (rat) fails to alternate between the two levers after being reinforced and continues to press the lever that produced the immediate food reinforcer (short-term memory error), and incorrect-lever perseverations. Perseverative behavior has been suggested to be an indicator of early AD (Traykov et al., 2005). This occurs when a subject continues to press the incorrect lever after making a lever-switching error (thus compounding the short-term memory error). This type of error indicates disruption of well-learned behaviors, or reference (long-term) memory, which includes reasoning and the goal-oriented manipulation of information.

When the ALCR schedule reflected no changes in operant response trends, the rats were anaesthetized using fentanyl citrate inhalation (0.4 ml/kg). They were placed in a stereotaxic frame (Kopf, USA), and fitted with a permanently indwelling cannula (23 gauge) aimed at the lateral cerebral ventricle. Half of the rats in each group received left lateral ventricle cannula implants and the other half received right lateral ventricle cannula implants. With the incisor bar set 3 mm below the interaural line, stereotaxic co-ordinates for cannula implantation were; bregma -1.0 mm, -1.5 mm lateral to the midline, and 3 mm below the pial surface (Paxinos and Watson, 1998). All rats were allowed 7 d for recovery before experimental testing. Prior to being assigned to a control or experimental group, the i.c.v. cannula placement and its patency was verified by vigorous drinking (>5 ml/20 min) following i.c.v. injection of 0.5 µg/ml of angiotensin II (Johnson and Epstein, 1974). The rats were then randomly assigned to control or experimental groups consisting of 12 animals per group. To establish response parameters in the absence of induction of a behavioral deficit through i.c.v. injection of 7PA2 CM, or of Bex administration, an overall control group was injected i.c.v. with CHO CM (wild-type control; 10 µl), then after 1 h was injected subcutaneously (s.c.) with 1% DMSO, and 1 h later tested using the ALCR schedule. A second control group, against which Bex effects would be measured, was injected i.c.v. with

7PA2 CM (10 µl), then injected s.c. after 1 h with 1% DMSO, and 1 h later tested using the ALCR schedule. This temporal sequence for behavioral testing 2 h following i.c.v. injection of 7PA2 CM has been previously described (e.g., Cleary et al., 2005). For the determination of Bex effects, separate groups were injected i.c.v. with 7PA2 CM (10 µl), then injected s.c. after 1 h with 2.5, 5, 10, or 15 mg/kg Bex. One h later they were tested using the ALCR schedule. Data were analyzed by one-way ANOVA, followed by Fisher's Post-Hoc Least Significant Differences tests.

2.5 *Transgenic Mice*

5XFAD (APPSwFILon, PSEN1*M146L*L286V) (B6JSL) mice were purchased from The Jackson Laboratory (Bar Harbor, ME, USA). The mice were divided into two groups. The control group (n=7) were fed normal chow and the test group (n=8) were fed chow containing 500 mg/kg Bex starting at 7-8 weeks of age, when Aβ deposits first begin to accumulate. Based on mouse weight and average food consumption, this corresponded to an oral dose of 2 mg per day, or 100 mg/kg/day. One mouse from each group was sacrificed at 20 days and one at 30 days for assessment of the accumulation of Aβ deposits. The remainder was sacrificed 48 days after commencement of Bex feeding. One week prior to sacrifice, animals from both groups were assessed for memory-related effects using a standard water maze test.

2.6 *Water maze test*

The water maze was set up in a pool 1.5 meters in diameter. A 10 cm diameter platform was placed in the south eastern quadrant of the pool. The mice were placed in the pool and required to swim to the platform. The training procedure consisted of a one day test where the platform was visible, followed by 4 days where an opaque fluid was used, so that

the platform was invisible. In the visible platform test, the mice received 5 contiguous trials, with an inter-trial interval of 30 min. In the hidden platform test, the mice were trained for 6 trials, with an inter-trial interval of 1 h. A probe trial was carried out 24 h after the last hidden platform test. Tracking of mouse movement was conducted with ANY-maze AVI recorder (Stoelting Co., IL, USA). Data were analyzed by two-way ANOVA.

2.7 *Mouse immunohistichemistry*

Immunohistochemistry to detect Aβ deposits was carried out using methods previously described (Guo et al., 2010). Briefly, cryostat sections approximately 16 microns thick were cut from paraformaldehyde fixed brains. They were treated for 5 min with 88% formic acid (Sigma-Aldrich) to enhance Aβ immunostaining. They were then treated for 15 min with 1% hydrogen peroxide to eliminate endogenous peroxide. This was followed by the addition of 5% skimmed milk and 2% normal rabbit serum, to block non-specific immunostaining. Sections were incubated with primary antibody overnight at room temperature. The primary antibodies were 6F/3D (Dako; 1:200) or anti-$A\beta_{42}$ (Mori; 1:2,000). This was followed by treatment with rabbit anti-mouse biotinylated IgG (Dako; 1:2000) for 1-h at room temperature. The sections were then developed by treatment with the ABC Vector kit (1:2000) for 1 h, followed by incubation in 0.01% 3,3 diaminobenzidine (DAB, Sigma, St Louis MO) containing 1% nickel ammonium sulfate and 0.001% hydrogen peroxide. After development of a deep purple color, the sections were washed and mounted.

3. **Results**

Fig. 1 shows the in vitro LTP of fEPSPs in the CA1 region of hippocampal slices. They were unaffected following the application of 20 μM Bex. Control LTP was 162.2 ± 8.9% after DMSO application, compared to an LTP of 165.1 ± 12.2% after Bex application. Fig. 2

shows that Bex was also ineffective in reducing the deficit in LTP caused by application of 7PA2 CM to hippocampal slices. Following HFS of the Schaffer collaterals, control LTP of 181.8 ± 9.2% of baseline fEPSP amplitude was unaffected by administration of CHO CM, with LTP of 171.6 ± 3.0% of baseline. However, in the presence of 7PA2 CM the amplitude of fEPSPs following HFS was reduced to 108.7 ± 8.2% of baseline. This deficit in LTP was unaffected following co-incubation of 7PA2 CM with Bex, the magnitude of LTP amounting to 101 ± 2.4%.

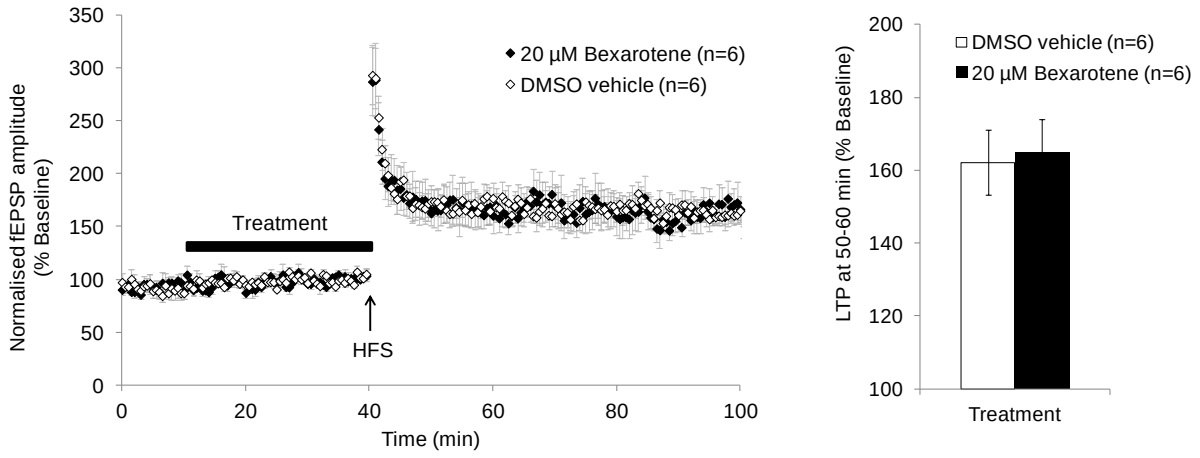

Fig. 1. Effect of Bex (20 µM) administration on fEPSP amplitude over time (left), and LTP at 50-60 min (right). Data are presented as mean±SEM.

None of the rats in the group injected s.c. with 15 mg/kg Bex produced sufficient operant output for analysis in the experimental chambers (a marked degree of general lethargy was also observed for these rats while in their home cages). Consequently, the behavioral analysis incorporating s.c. injection of Bex at 2.5, 5, and 10 mg/kg indicated that statistically there was a significant overall treatment effect on lever-switching errors ($F_{5,71}$=2.812, p=0.02; Fig. 3). The group injected i.c.v. with 7PA2 CM and injected s.c. with vehicle exhibited significantly more lever-switching errors than the group injected i.c.v. with CHO CM and

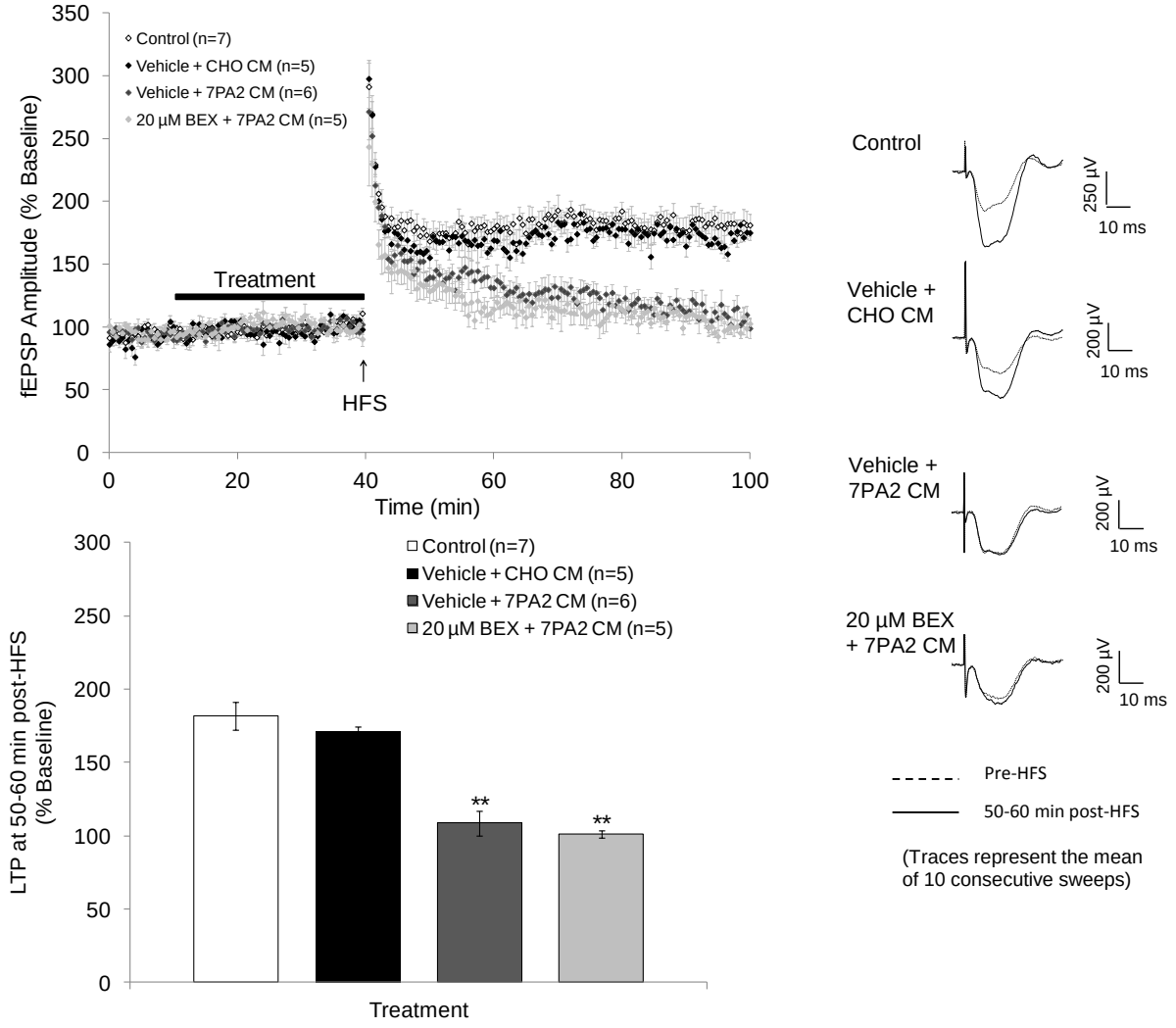

Fig. 2. Effect of Bex (20 µM) administration on deficits in LTP induced by 7PA2 CM. Shown are fEPSP amplitude over time (top left), LTP at 50-60 min (bottom left), and typical traces from a single experiment per group set (right). Data are presented as mean±SEM. **P<0.0001 indicates significant differences compared to CHO CM + vehicle.

injected s.c. with vehicle (p<0.03). This confirmed the behavioral deficit induced following i.c.v. 7PA2 CM injections. However, there were no significant differences in lever-switching errors between the i.c.v. 7PA2 CM injected group given s.c. vehicle (DMSO) injection and the i.c.v. 7PA2 CM injected groups given s.c. Bex injections at 2.5, 5, and 10 mg/kg (p's>0.5). In fact, s.c. Bex administration dose-dependently increased the effect size of lever-

switching errors (p=0.04, p=0.03, p=0.001), suggesting that Bex might be dose-dependently increasing the behavioral deficit rather than decreasing it.

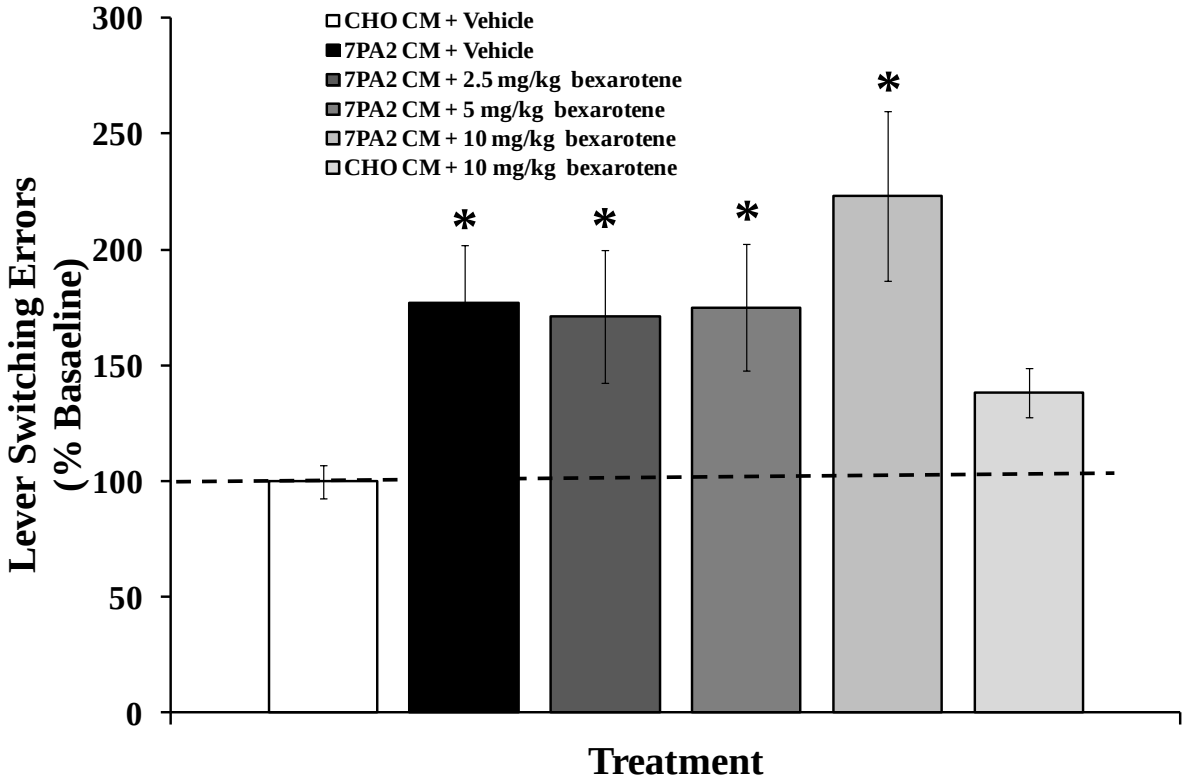

Fig. 3. Effect of Bex administration on i.c.v. 7PA2 CM-induced lever switching errors. Data are presented as mean±SEM. *P<0.05 indicates significant differences CHO CM + vehicle as compared to the 7PA2 CM + vehicle, 2.5 mg/kg, 5 mg/kg and 10 mg/kg Bex.

With respect to incorrect lever perseverations, there was a significant overall treatment effect ($F_{5,71}$=2.538, p<0.03; Fig. 4). The group injected i.c.v. with 7PA2 CM and injected s.c. with vehicle exhibited significantly more incorrect lever perseverations than the group injected i.c.v. with CHO CM and injected s.c. with vehicle (p<0.028). However, there were no significant differences in incorrect lever perseverations between the i.c.v. 7PA2 CM injected group given s.c. vehicle (DMSO) injection and the i.c.v. 7PA2 CM injected groups

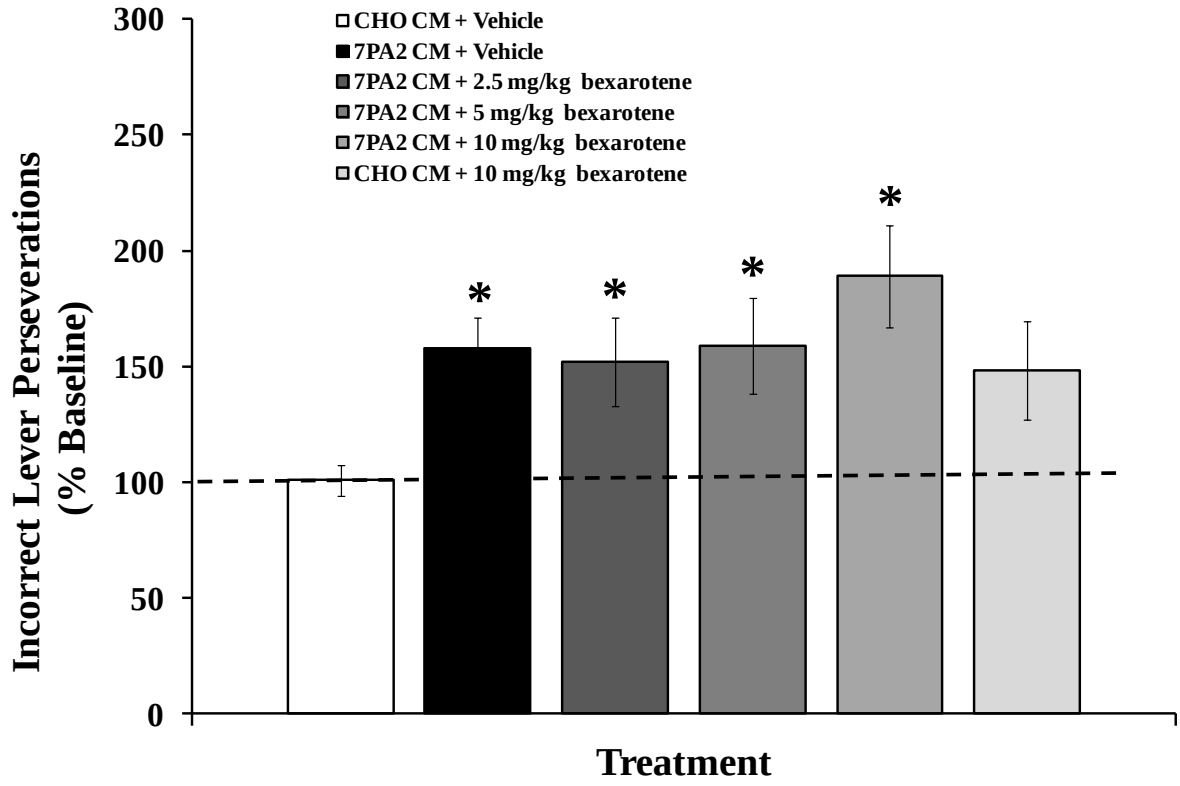

Fig 4. Effect of Bex administration on i.c.v. 7PA2 CM-induced incorrect lever perseverations. Data are presented as mean±SEM. *P<0.05 indicates significant differences CHO CM + vehicle as compared to the 7PA2 CM + vehicle, 2.5 mg/kg, 5 mg/kg and 10 mg/kg Bex.

given s.c. Bex injections at 2.5, 5, and 10 mg/kg (*p*'s>0.5). And again, s.c. Bex dose-dependently increased the effect size (p=0.04, p=0.02, p=0.001).

Fig. 5 shows the results of the water maze test performed on the B6SJL mice. All mice improved their performance with training. However there was no significant improvement in escape latency of Bex fed mice compared with control mice. In fact they performed worse in 60 seconds. In the spatial memory test for platform location, no improvement of Bex fed mice was observed. Fig. 6 shows typical immunohistochemical results of Abeta deposits in control and Bex fed mice. They show extensive deposits in both control and Bex fed mice with no evident sparing by Bex feeding. The numbers of deposits were counted in

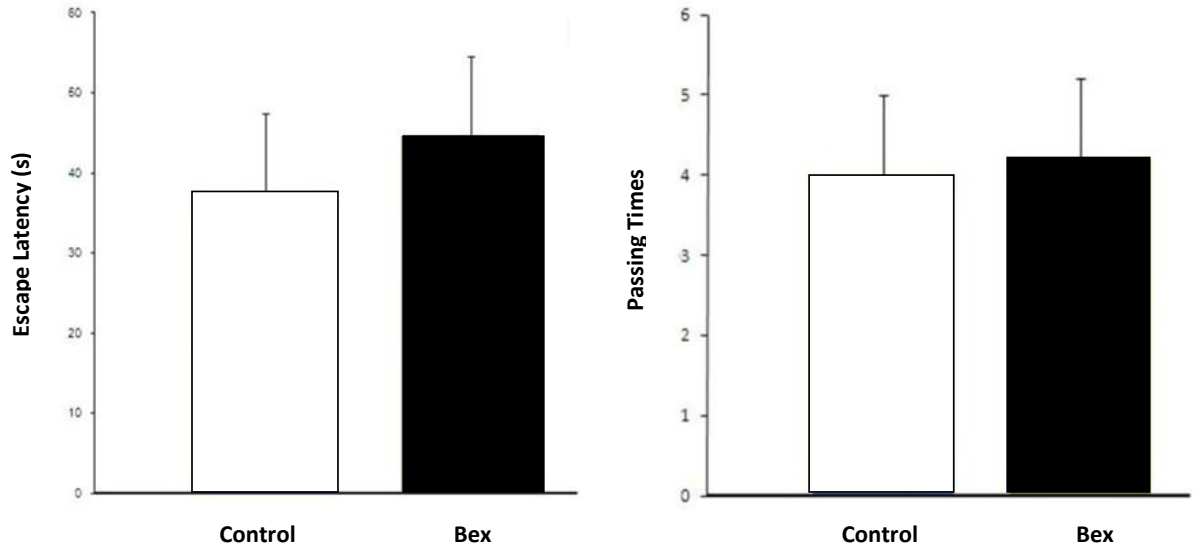

Fig 5. Water-maze behavioural testing in Bex treated and control (non-Bex treated) B6SJL transgenic mice.

representative fields to give a semi-quantitative assessment of the deposits. The plaques were counted by computer-aid program Image J. The number detected with the 6F/3D antibody were: Bex:1306+/-211, CTL: 1397+/-162. The number detected with the anti-$A\beta_{42}$ was: Bex: 941+/-143, CTL: 879+/-105.

4 **Discussion**

Cramer and colleagues (2013) reported that oral administration of Bex in Tg mice produced enhanced clearance of soluble Aβ within hours. They reported that Aβ plaques were reduced by more than 50% within 72 h, and that there was rapid reversal of cognitive deficits and improved neural circuit function. Even though four independent research groups (Fitz et al., 2013; Veeraraghavalu et al., 2013; Price et al., 2013; Tesseur et al., 2013) could not fully reproduce these findings, Landreth and colleagues (2013) responded that the overall findings of these independent groups replicated and validated the central conclusions of the Cramer et al. (2013) paper.

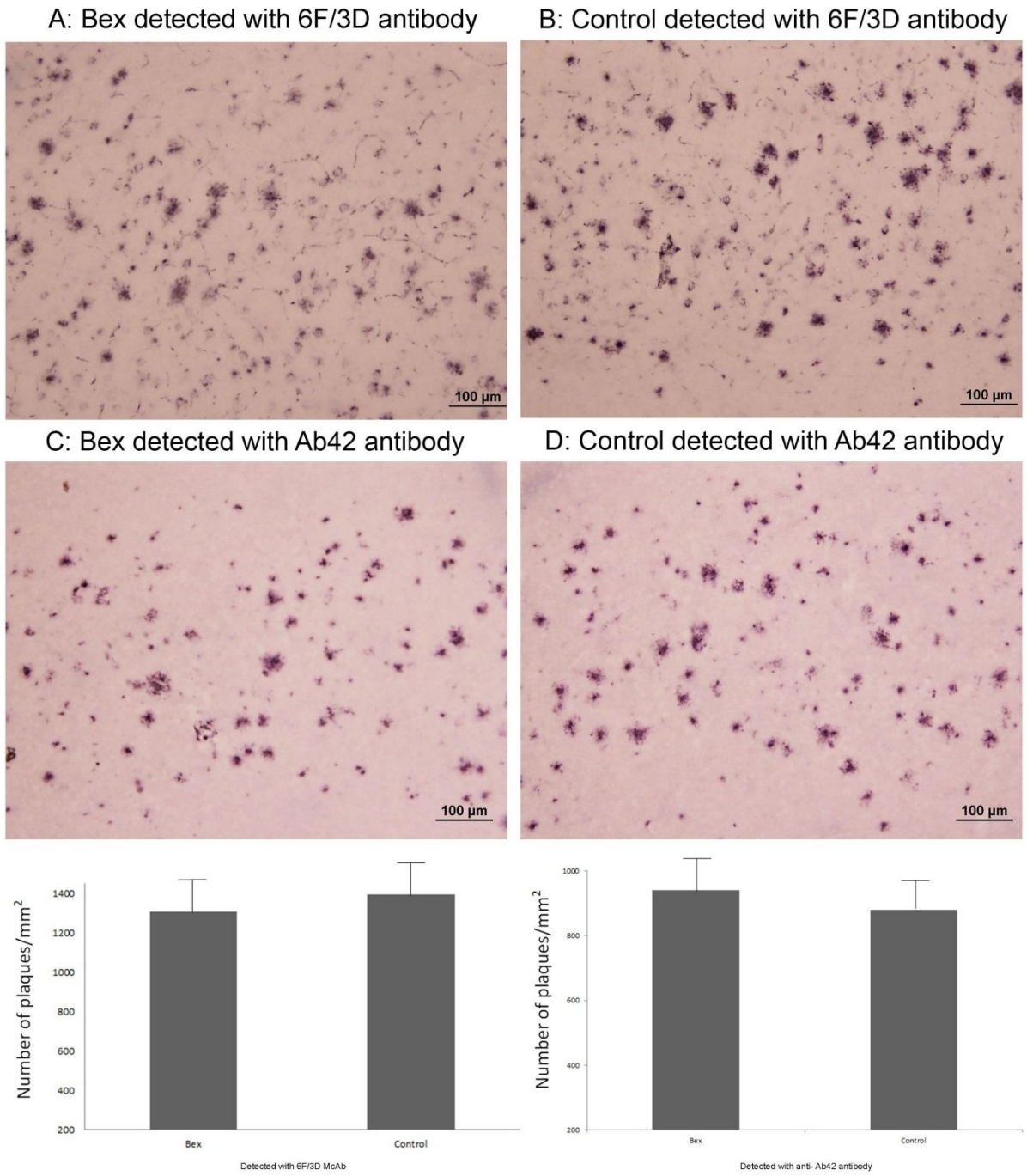

Fig. 6. Immunostained sections illustrating plaque accumulation and plaque counts in the cerebral cortex of Bex treated and control (non-Bex treated) B6SJL transgenic mice. Bex treatment was commenced at 6-7 weeks of age and continued for 48 days. Immunostaining was with 6F3D which recognizes $A\beta_{40}$ and $A\beta_{42}$, and a specific $A\beta_{42}$ antibody which recognizes only the latter peptide. There was no observable effect of Bex.

However, none of the independent research groups investigated the electrophysiological effects of Bex. LTP provides an experimental representation of memory at the level of the synapse when applied to hippocampal synaptic events (Bliss and Collingridge, 1993). Moreover, operant behavior analytical techniques are highly sensitive (Skinner, 1963). The rat offers a greater brain area for the collection of detailed electrophysiological data, and historically rats have been the rodent of choice where behavioral effects have been important (e.g., Ferster and Skinner, 1957). Also, the rat has been first choice when models incorporating accurate behavioural measurement relative to pharmacological manipulations have been a crucial area of investigation (e.g., Ferster and Skinner, 1957; Wells and Carter, 2001; Cenci et al., 2002). For these reasons, our study used normal rats for the measurement of electrophysiological and behavioral effects following Bex administration. We found no beneficial effects of Bex, even at high s.c. doses.

Cramer et al. (2013) also reported that Bex restored cognition and memory in APP/PS1 mice, as assessed using a contextual fear conditioning task, when administered for 7 days at the 6 and 11 month stages of plaque pathogenesis, and when administered for 90 days and analysed when the mice were 9 months old, and also in APPPS1-21 mice at 7-8 months following Bex administration for 20 days. They also reported that APP/PS1 mice administered with Bex for 90 days, and APPPS1-21 mice administered with Bex for 20 days exhibited enhanced performance when assessed using a water maze task.

In contrast to these findings, we found no beneficial effects of Bex in a water-maze test using the B6SJL mouse model of AD (Fig. 5). To enhance the possibility of a favorable effect, the high oral dose of 100 mg/kg/day was employed and the dose extended to 48 days of administration. This negative finding is consistent with our observation that Bex had no ameliorative effect on the accumulated deposition of Aβ deposits (Fig. 6). Again, to enhance the possibility of a positive effect, Bex administration was commenced at the time when

deposits first start to accumulate. By adopting this approach, any preventative effect of Bex administration should have been apparent.

Bex is not without side effects in humans, according to its Federal Drug Administration (FDA) approval status (Lowednthal et al., 2012). The FDA cautions that Bex administration to humans causes lipid abnormalities, alters the action of insulin in diabetic patients, and is associated with hypothyroidism, leukopenia, elevated liver function values, and an increased risk for acute pancreatitis. Consequently, the potential value of Bex administration for the management of AD, using the application of varying pre-clinical models, is a matter of significant importance prior to the advocacy of this drug for clinical trials in humans suffering from AD. The reported results, employing two varying rodent models of AD, indicate negative effects on electrophysiological and behavioral outcomes in the rat, and indicate no positive effect on behavior or histology using an aggressive model of AD in the Tg mouse. In conclusion, the results of the pre-clinical animal studies reported here, proffer no support with respect to the putative therapeutic effects of Bex in human clinical trials.

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